Isolation of the Capsular Polysaccharide from Culture Supernatant of *Haemophilus influenzae* Type b

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The capsular polysaccharide (CP) of *Haemophilus influenzae* type b is known to be spontaneously released from the cells in culture. The CP is precipitable from culture supernatant by the cationic detergent hexadecyltrimethylammonium. Most of the nucleic acid and some of the protein, but almost none of the endotoxin, in the supernatant are co-precipitated. Extraction of the precipitate with progressively stronger NaCl solutions separates nucleic acid and protein from the CP and also effects a molecular size fractionation. Residual endotoxin and protein can be reduced by extraction with cold phenol and ultracentrifugation. The resulting preparation has ribose, ribitol, and phosphate as principal components and contains less than 1% other sugars, protein, or nucleic acid; it elutes on Sepharose 2B as a symmetrical peak with K_{av} 0.51.

The capsular polysaccharide antigen (CP) of *Haemophilus influenzae* type b was characterized as a polymer of D-ribose phosphate and named polyribophosphate (25). Subsequent studies identified ribitol as an additional constituent (4) and proposed a structure based upon equimolar ribose, ribitol, and phosphate (6). CP appears to be the principal virulence determinant in systemic infections by *H. influenzae* type b (1), and recent interest in its potential as a protective immunogen (17, 22) has prompted efforts to improve purification methods.

Early approaches to CP isolation used fractional precipitation of liquid culture filtrates with organic solvents. Multiple precipitations (7) or electrophoresis (25) was used to separate the antigen from other similarly precipitable materials in the cultures. Zamenhof and Leidy (Fed. Proc. 13:327, 1954) extracted CP from cells suspended from solid medium, thereby reducing the working volume and eliminating most of the potential contaminants from the medium itself; sodium dodecyl sulfate was used to reduce protein and charcoal was used to adsorb nucleic acids. From cells prepared similarly, Rodrigues et al. isolated CP by ion-exchange and chloroform extraction (18). Studies in this laboratory have shown that CP is synthesized throughout growth and in early stationary phase, by which time, however, most of the antigen has been released into the medium (3). Optimal yield thus requires isolation of CP from culture supernatants, which contain nu-

merous other bacterial materials as well as components of the complex media required for *H. influenzae* type b.

The use of precipitation by quaternary nitrogen detergents for isolation of an anionic polysaccharide was introduced by Jaques et al. (11) and extensively developed by Scott (21), primarily with polymers of animal and plant tissues. The principle has since been used for various microbial polysaccharides, including the isolation of the capsular polysaccharides from whole cultures of *Neisseria meningitidis*, serogroups A, B, and C (9). The latter methodology has been directly applied to isolation of CP from *H. influenzae* type b (J. Robbins, personal communication). The present report describes the isolation of CP from cell-free culture supernatants of *H. influenzae* type b by a combination of detergent precipitation and selective extraction that also effects a molecular size fractionation of the antigen.

MATERIALS AND METHODS

The strains used and their growth and CP elaboration have been described (2, 3). Base media were brain heart infusion (Bioquest, Cockeysville, Md.) or CY medium, which consists (per liter) of 10 g of Casamino Acids (Difco, Detroit, Mich.), dialysate of 5 g of Difco yeast extract, 5 g of glucose, and 0.1 mol of sodium phosphate buffer, pH 7.6. Base media were supplemented (per liter) with 1 mg each of nicotinamide adenine dinucleotide (Sigma Chemical Co., St. Louis, Mo.) and hemin (Eastman Kodak, Rochester, N. Y.), the latter freshly dissolved in 1 N NH₄OH at 3 mg/ml.

Cultures were 0.5 liter in 2-liter baffled Erlenmeyer flasks, shaken vigorously at 37°C until 8 to 10 h after the cessation of growth. At this point subcul-

tures to ascertain purity were made onto supplemented CY agar and onto blood agar (nonpermissive for *H. influenzae*), and 10 ml of formalin per liter was added; the cultures were held at 4°C overnight, and the subcultures were examined. Cell-free supernatant was then prepared by two cycles of centrifugation. Centrifugations were for 15 min at 10,000 × *g* at 0 to 5°C unless otherwise noted. Hexadecyltrimethylammonium bromide (HB; Eastman Kodak) was technical grade.

Pentose was assayed by the orcinol reaction (5) with D-ribose as standard, organic phosphate by the ammonium molybdate reaction (14), hexose and heptose by the cysteine-sulfuric reaction (24) with D-glucose and glucoheptose, respectively, as standards, and protein by the Folin phenol reagent (15) with bovine serum albumin as standard. Protein was also estimated along with nucleic acid from optical density at 280 and 260 nm and Adams' nomograph (California Corp. for Biochemical Research, Los Angeles). Sodium was determined by flame photometry (model 143, Instrumentation Laboratory Inc., Watertown, Mass.) with a lithium internal standard. Dry weight of CP was determined by lyophilization of an aqueous sample, followed by further drying to constant weight under vacuum over P_2O_5 at room temperature. CP-specific pentose was measured by immunochemical assay (3). Precipitating antigens were enumerated by Ouchterlony double diffusion as described (12) with rabbit antiserum to living exponential-phase *H. influenzae* type b, strain Eag. *Limulus polyphemus* amebocyte lysate (LAL) was prepared and used in an assay for endotoxin according to Rojas-Corona et al. (19); 10-fold dilutions of samples or standard were assayed. The standard was lipopolysaccharide from *Escherichia coli* O111:B4 (Difco), which caused full gelation at dilutions out to 8 ng/ml. Pyrogenicity was tested in rabbits.

Molecular sieve chromatography was done with Sepharose 4B or 2B (Pharmacia Inc., Piscataway, N.J.) in a 2.3-cm-diameter column packed to a height of about 50 cm with gel in phosphate-buffered saline (2). Void volume was determined by (the leading peak of) blue dextran 2000 (Pharmacia) and total volume by ^3H_2O. Samples containing 200 to 300 μg of pentose were applied in 1 ml. Elution was at 5 ml/cm² per h at 4°C.

Gas-liquid chromatography (GLC) was done in a Hewlett-Packard model 5830 A with the 6-foot (182.8-cm) column packed with SE-30 (Pierce Chemical Co., Rockford Ill.) and helium flow at 70 ml/min. Samples were heated for 4 h at 100°C in 4 N HCl, vacuum dried over P_2O_5 at room temperature, and derivatized with 0.25 ml of trimethysilylimidazole (Pierce) at 65°C for 15 min. (The acid hydrolysis was expected to cause some degradation of ribosyl residues.)

RESULTS

Experiments to determine precipitability by HB used CP prepared according to Zamenhof and Leidy (Fed. Proc. 13:327, 1954). The antigen dissolved in culture medium at 10 μg of pentose per ml was reacted with HB under various conditions, and the extent of precipitation was determined by pentose assay of the precipitate (Table 1). Precipitation increased with HB concentration through 0.01 M, occurred over a broad range of pH, and decreased as the temperature was raised from 0°C. These variables pertained equally in culture media made with brain heart infusion or Casamino Acids and yeast extract. HB, however, also precipitated ribonucleic acid (which is orcinol positive) from yeast extract-containing media; this complication, as well as the precipitation of Lowry-positive material from media, was reduced by the use of a dialysate medium (CY).

CP in the hexadecyltrimethylammonium (H) precipitate could be solubilized with salts of sodium, potassium, or calcium (no others tested); NaCl was used for most further study. Cell-free supernatant of a stationary-phase culture of *H. influenzae* b strain Eag in CY-based medium was reacted with HB, and the resulting precipitate was extracted sequentially with water and then with NaCl solutions of 0.1, 0.2, 0.25, 0.3, 0.4, and 2 M (extraction included mechanical dispersion, vigorous mixing, and centrifugation). The extracts were made at least 0.4 M in NaCl, mixed with 2 volumes of 95% ethanol, and held for at least 2 h at −20°C. The precipitates were extracted with water, and the

TABLE 1. *Effect of several variables on the precipitation by HB of purified CP dissolved in uninoculated culture medium* [a]

Variable	Value	% Pentose precipitated
HB concentration (mM)	5	15
	10	81
	20	86
	40	84
pH [b]	9.0	45
	7.0	85
	5.0	76
	3.0	80
	2.5	70
	2.1	47
	1.7	27
Temperature (°C)	0	89
	25	64

[a] The medium was brain heart infusion supplemented with nicotinamide adenine dinucleotide and hemin as described. (As supplied, the basal medium contains 5 g of NaCl and 2.5 g of Na_2HPO_4 per liter, pH 7.4.) CP was added at 10 μg of pentose per ml. HB was added at 10 mM, and the temperature was 0°C throughout, except where noted. After incubation for 30 min the mixtures were centrifuged, and the extent of CP precipitation was determined by pentose assay of the precipitates, with corrections made for controls, to which no CP was added.

[b] Adjusted with NaOH or HCl.

extracts were assayed (Table 2). About 80% of the CP pentose that had been present in the culture supernatant was recovered, mainly in the first four NaCl extracts. Only 10% of the (Lowry) protein was recovered, primarily at higher molarities. Protein as estimated by optical density at 280/260 nm was higher than the Lowry values in the various extracts but had a similar proportional distribution. Nucleic acid (estimated by optical density) was about 80% recovered in the extracts, mainly at higher molarities. The LAL assay for endotoxin was positive at a titer of 10,000 in the culture supernatant; only about 1% of the activity was recovered in the extracts, mainly in the first two NaCl extracts.

The Sepharose 4B profiles of CP in the various NaCl extracts are shown in Fig. 1. CP in the 0.1, 0.2, and 0.25 M extracts was seen in symmetrical peaks of increasing apparent size. CP in the 0.3 M extract appeared even larger, consisting of a peak of K_{av} 0.17, but with a majority of material eluting in the void volume.

The scheme of precipitation with HB and differential extraction with NaCl were carried out with six separate cultures of strain Eag and one each of strains Rab, Mad, and 305, all in CY-based medium. In every case increasing NaCl concentrations gave extracts in which the apparent size of the CP was greater and protein content was higher.

In separate experiments the effect of pH upon the differential precipitation by HB of the polysaccharide and protein was explored. Samples of culture supernatant of strain Eag containing 0.1 M sodium phosphate buffer were adjusted with HCl to pH 7, 5, 4, or 3, and HB was added at 0.01 M. The resulting precipitates were extracted with 2 M NaCl, and the ethanol-precipitable fraction of the extracts was assayed for CP pentose and protein. Recovery of CP was about

90% at each pH, and the recovery of protein was also equal; i.e., no differential was found.

The material from the 0.2, 0.25, and 0.3 M extracts described in Table 2 and Fig. 1 was pooled for further purification by extraction with phenol at 4°C, reprecipitation from 0.4 M NaCl by ethanol, dialysis against water, and removal of a small amount of proteinaceous material by sedimentation for 2 h at approximately 100,000 × g. Data on composition (Table 3) indicate an equimolar ratio of pentose, organic phosphate, and sodium. Colorimetric assay indicated the presence of trace amounts of hexose and heptose. Activity in the LAL assay, although about 100-fold lower than in the pooled NaCl extracts, was still detectable.

Composition of the CP was further explored by GLC of trimethylsilyl derivatives of acid hydrolysates. Figure 2A shows a standard mix-
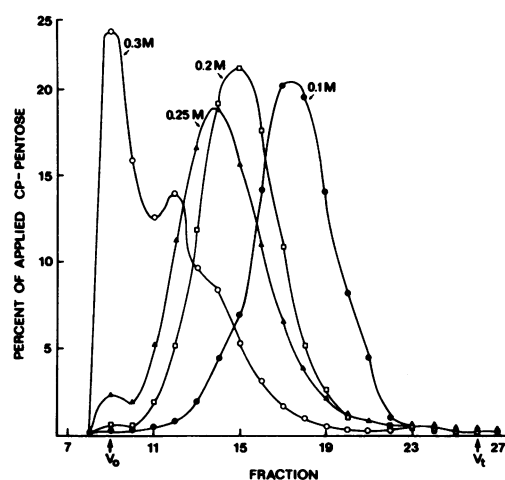

FIG. 1. Profile on Sepharose 4B of CP in fractions extracted from H precipitate with a series of increasing NaCl concentrations.

TABLE 2. Assays for CP and other materials in culture supernatant and in extracts of its precipitate with HB

Sample	μg/ml of original culture				Non-CP precipitin lines	LAL titer[c]
	CP pentose	Protein[a]	Protein[b]	Nucleic acid[b]		
(1) 20,000 × g culture supernatant[b]	37	47	58	5.1	4	10,000
(2) Water extract of HB precipitate of (1)	<0.50	0.49	1.5	0.14	0	1
(3) 0.1 M NaCl extract after (2)	3.8	0.014	0.055	0.0038	0	100
(4) 0.2 M NaCl extract after (3)	10	0.22	0.86	0.075	0	100
(5) 0.25 M NaCl extract after (4)	6.8	0.58	1.2	0.17	0	10
(6) 0.3 M NaCl extract after (5)	7.9	1.9	3.8	0.65	1	10
(7) 0.4 M NaCl extract after (6)	0.30	1.1	2.3	0.40	1	10
(8) 2 M NaCl extract after (7)	0.36	0.41	8.8	2.8	0	1

[a] By Lowry assay (15).
[b] Estimated from optical density at 280 and 260 nm with a nomograph; not corrected for light scattering.
[c] Reciprocal of greatest dilution giving full gelation.
[d] A small sample dialyzed to facilitate the estimation of protein and nucleic acid.

TABLE 3. *Composition and other data on purified CP*

Assay	Value
Pentose	2.68 μmol/mg^a (40.2%)
Organic phosphate	2.70 μmol/mg (17.0%, as PO$_2$)
Sodium.................	2.36 μmol/mg (4.9%)
Hexose	0.023 μmol/mg (0.41%)
Heptose	0.010 μmol/mg (0.21%)
LAL end point^b	4.5 μg/ml
Protein (Lowry).........	5.3 μg/mg (0.53%)
$A_{280}^{1\%}$^{c}	0.65
$A_{260}^{1\%}$	1.10
K_{av}, Sepharose 2B	0.51

^{a} Based on dry weight.

^{b} Least concentration giving full gelation.

^{c} A, Absorbancy.

ture of (acid-treated) ribose, ribitol, and glucose. The complex of peaks at 3 to 3.5 min was produced by ribose, the peak at 4.6 min by ribitol, and the peak at 2 min by the product of the action of HCl on ribitol (presumably 1,4-anhydroribitol); glucose produced the triad at 7 to 9 min (alpha anomer) and the peak at 13 min (beta anomer). The hydrolysate of the CP pool prior to phenol extraction (Fig. 2B) gave major peaks indicating the presence of ribitol and, as expected from the hydrolytic conditions, a small ribose complex; there were also minor peaks at 8 to 9 min, 13 min, and (possibly) 17 min. The hydrolysate of phenol-purified CP (Fig. 2C) gave peaks characteristic of ribitol and ribose, barely discernible peaks at 8 to 9 and 13 min, and two very small peaks not identified.

The molecular sieve profile of the purified CP, because it contained material eluting in the void volume of Sepharose 4B, was determined on Sepharose 2B. It eluted as a symmetrical peak with K_{av} 0.51, with only about 1% eluting in the void volume.

DISCUSSION

The methodology of Gotschlich et al. for isolation of meningococcal CPs (9) had the welcome result of generating immunologically quite active preparations of great value in the study of the host response to and vaccination against this pyogenic gram-negative species. The isolation procedure began with the addition of HB to whole cultures, one potential effect of which is that the detergent acts upon the membrane to release cellular materials not otherwise present in the supernatant. In undertaking the somewhat analogous goal of isolating CP from *H. influenzae* type b, we found in bacteriological experiments that essentially all

of the antigen is freely released in culture within 6 to 8 h after synthesis (3). Addition of HB to the culture in concentration sufficient to precipitate the CP was found to cause the release of a fair quantity of macromolecular material that precipitated along with the polysaccharide. It thus seemed advantageous to add the detergent to cell-free supernatant and thereby simplify subsequent purification steps.

Under the conditions used, at least 80% of the CP was precipitated from culture supernatant along with a similar proportion of the superna-
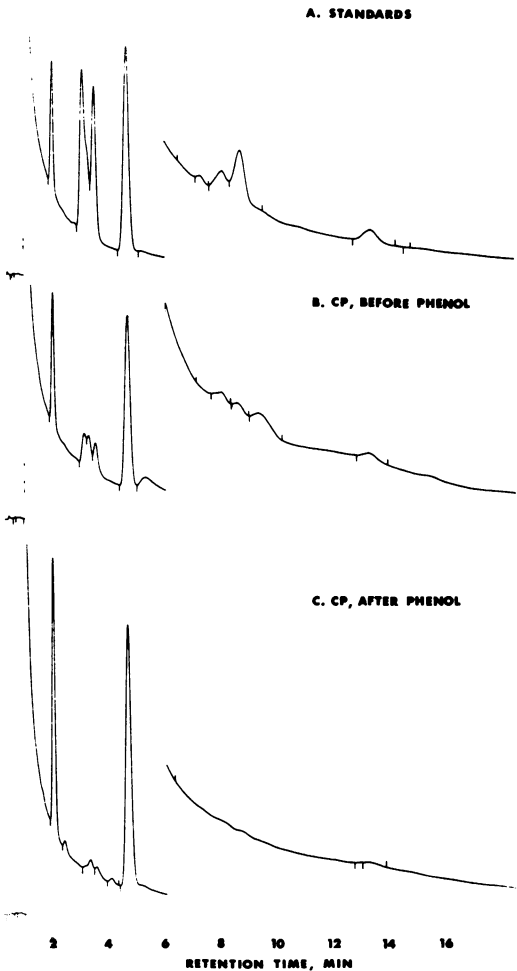

FIG. 2. *Gas-liquid chromatograms of standards and CP before and after phenol extraction. Samples were: A, Mixture of 0.5 μmol of ribitol, 0.5 μmol of D-ribose, and 0.01 μmol of D-glucose. B, CP prior to phenol extraction; sample contained 0.5 μmol of pentose. C, CP after phenol extraction and reprecipitation from ethanol; sample contained 0.5 μmol of CP pentose. The break in the trace at 6 min is a shift in attenuation, for greater sensitivity.*

tant nucleic acid. The precipitability of nucleic acids by quaternary nitrogen detergents has long been established; similar behavior of CP was a predictable result, since both contain a high multiplicity of phosphoric acid groups. Only about 10% of the supernatant protein was extracted from the H precipitate; the data do not indicate whether more protein may have been precipitated, but in a form not extractable by 2 M NaCl (the highest concentration compatible with the ethanol precipitation). From principles described by Scott (21), we expected that precipitation at the lowest pH at which secondary phosphates are fully charged would reduce the ratio of protein to CP by excluding all but the most acidic proteins; however, no selectivity was evident in the range of pH 7 to 3.

Extraction of the H precipitate with progressively higher salt concentrations is a "batch" procedure roughly equivalent to ion-exchange chromatography. CP could be solubilized selectively from most of the precipitated protein and nucleic acid. Moreover, an apparent size fractionation of the CP was found: the molecules solubilized at lower salt concentrations eluted later from Sepharose 4B. That size rather than composition was the determinant is suggested by the finding that faster and slower eluting fractions did not differ in phosphate content. A dependence of solubilizing salt concentration upon a polymer's size was noted by Scott (21) and used preparatvely by Laurent for molecular weight fractionation of heparin (13).

The 0.3 M NaCl extract contained a substantial proportion of the larger-sized CP but was somewhat contaminated with nucleic acid and protein. Thus, additional methods for nucleic acid removal were examined. Activated charcoal (Zamenhof and Liedy, Fed. Proc. 13:327, 1954) adsorbed nucleic acid satisfactorily; however, quite stable colloidal suspensions of charcoal in the CP solution sometimes resulted. In purifying the meningococcal polysaccharides, Gotschlich et al. (9) extracted the H precipitate with $CaCl_2$ and removed nucleic acids from the extract by fractional precipitation with ethanol. This procedure with *H. influenzae* type b extracts did indeed reduce nucleic acid content, but also resulted in substantial losses of CP. Without additional purification procedures, however, the nucleic acid content of CP preparations was kept low by the use of dialysate medium and by minimizing spontaneous cell lysis. Residual protein in the NaCl extracts was readily reduced by cold phenol extraction, with no loss of CP.

Lipopolysaccharide (LPS) prepared by hot phenol extraction (23) of *H. influenzae* type b contains hexoses and heptose and is active in the LAL assay in concentrations as low as 5 ng/ml (A. Flesher, personal communication). Although materials other than LPS can be LAL positive (8), it is likely that LPS spontaneously released from the cells was responsible for the substantial LAL activity of the culture supernatant and the (much lesser) activity of the CP preparation. This account is supported by the GLC profile (Fig. 2B), which includes a pattern of small peaks at 8 to 9 and 13 min characteristic of hexose or hexosamine and a slight elevation at 17 min suggesting a heptose, as well as by colorimetric assay (Table 2). Only about 1% of the supernatant's LAL activity was extractable from the H precipitate. Probably the active material was soluble in the presence of HB but was occluded in trace amounts in the precipitate. This explanation is supported by separate experiments showing that the hot phenol-purified LPS was not precipitated by HB under the conditions used for CP. It is likely that CP could be further freed of LPS contamination by repeated cycles of HB precipitation/NaCl extraction. A similar differential in the HB precipitability of LPS and capsular polysaccharide has been observed with enteric bacilli (16, 23). Interestingly, extraction of the CP with cold phenol appeared also to further reduce the LPS content, as seen in a reduction both of LAL activity and of the GLC peaks characteristic of hexose (cf. Fig. 2B and C). This unexpected finding might have one of the following explanations: (i) some of the LPS was tightly bound to protein or other phenol-extractable material; (ii) the LPS, although hydrophilic, was aggregated by the phenol treatment into particles sedimented to the phenol-water interface by the centrifugation at $10,000 \times g$; or (iii) materials other than LPS were responsible for the residual *Limulus* activity and minor GLC peaks.

The composition of this CP preparation is very similar to that described by Zamenhof et al. (25) and to our preparations from strain Eag made by their methods. Precise determination of the ribitol content is complicated by its alteration during acid hydrolysis of the polymer. In the GLC profiles, however, the ribitol-associated peaks generated from the CP sample containing 0.5 μmol of ribose are similar in size to those from the standard mixture containing 0.5 μmol of ribitol. This observation and the weight not accounted for by sodium, phosphate, and ribose moieties (37% of total; Table 3) are consistent with the equimolar ribose/ribitol ratio proposed by Crisel et al. (6).

The immunogenicity of a bacterial polysaccharide may vary with molecular weight (10) and with residual content of immunopotentiating agents such as LPS (20). Ideally, such fac-

tors should be defined and perhaps varied in a controlled fashion in the evaluation of the vaccine potential of a given polysaccharide. The methods described here for isolation and fractionation of CP permit a modicum of definition; also, being technically simple and adaptable to bulk processing, they could serve as preliminary steps to more sophisticated biochemical separation procedures.

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